

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Ken-ichi Arai et al.

Title: NF-AT DERIVED POLYPEPTIDES THAT BIND  
CALCINEURIN AND USES THEREOF

Appl. No.: To be assigned

Filing Date: April 14, 2000

**UTILITY PATENT APPLICATION TRANSMITTAL**Assistant Commissioner for Patents  
Box PATENT APPLICATION  
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(b) is the nonprovisional  
utility patent application of:Ken-ichi Arai  
Jie Liu

Enclosed are:

- ☒ Specification, Claim(s), and Abstract (42 pages).
- ☒ Informal drawings (11 sheets, Figures 1-11).
- ☒ Preliminary Amendment

The filing fee is calculated below:

	Claims as Filed	Included in Basic Fee	Extra Claims	Rate	Fee Totals
Basic Fee				\$690.00	\$690.00
Total Claims:	14	- 20	= 0	x \$18.00	= \$0.00
Independents:	2	- 3	= 0	x \$78.00	= \$0.00
If any Multiple Dependent Claim(s) present:			+ 0	\$260.00	= \$0.00
				SUBTOTAL:	= \$690.00
<input checked="" type="checkbox"/> Small Entity Fees Apply (subtract ½ of above):					= \$345.00
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**23533**

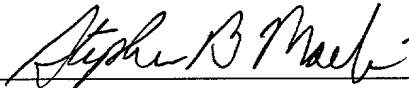
PATENT TRADEMARK OFFICE

- [ X ] The required filing fees are not enclosed but will be submitted in response to the Notice to File Missing Parts of Application.
- [ ] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Assistant Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date APR 14 2000

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**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully request that the following amendment be entered into the application:

**In the Claims:**

Please rewrite claim 5 as follows:

5. (Amended) A method for producing the polypeptide of claim 1, [the method comprising culturing the transformant of claim 4,] and recovering the expressed polypeptide from the transformant or the culture supernatant.

Please add new claim 14:

--14. A method comprising culturing the transformant of claim 4, and recovering the expressed polypeptide from the transformant or the culture supernatant.--

**REMARKS**

Applicants respectfully request that the foregoing amendment to claim 5 be entered in order to avoid this application incurring a surcharge for the presence of one or more multiple dependent claims.

Respectfully submitted,

Date APR 14 2000

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## NF-AT DERIVED POLYPEPTIDES THAT BIND CALCINEURIN AND USES THEREOF

## FIELD OF THE INVENTION

This invention relates to novel polynucleotides, polypeptides  
5 encoded by them, use of the polynucleotides and polypeptides, and  
a method for producing them. More particularly, the present  
invention relates to Calcineurin (CN)-binding polypeptides.

## BACKGROUND OF THE INVENTION

10 The calcium/calmodulin-dependent serine/threonine phosphatase, Calcineurin (CN) (Masuda, E. S. et al., 1999, Cell. Signaling 10:599), is a heterodimeric protein composed of a calmodulin-binding catalytic subunit, CNA, and a  $\text{Ca}^{2+}$ -binding regulatory subunit, CNB (Kincaid, R. 1993, Adv. Second Messenger  
15 Phosphoprotein Res. 27:1). CN is a target of the immunosuppressive drugs cyclosporin A (CsA) and FK506, which block T cell function by preventing transcriptional activation of cytokine genes. It has been suggested that CN plays an essential role in calcium-dependent dephosphorylation signal transduction pathways and  
20 subsequently leads to production of cytokines in T cells (Crabtree, G. R., and N. A. Clipstone, 1994, Annu. Rev. Biochem. 63:1045). Further studies have revealed that the potential of CN to regulate the expression of cytokine genes is largely due to effects on activation of a transcription factor termed nuclear factor of  
25 activated T cells (NF-AT) (Rao, A. et al., 1997, Annu. Rev. Immunol. 15:707; Masuda, E. S. et al., 1999, Cell. Signaling 10:599).

Currently, five NF-AT family members (NF-AT1/NF-ATp, NF-ATc/NF-AT2, NF-ATx/NF-AT4/NF-ATc3, NF-AT3, and TonE-BP/NF-AT5) have been identified, and they share functional and structural similarities (Hoey, T. et al., 1995, Immunity 2:461; Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15:2697; McCaffrey, P. G. et al., 1993, Science 262:750; Northrop, J. P. et al., 1994, Nature 369:497; Miyakawa, H. et al., 1999, Proc. Natl. Acad. Sci. U S A 96: 2538-42;

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Mol. Cell. Biol. 17:2066; Beals, C. R. et al., 1997, Genes Dev. 11:824), and another functional NLS (Beals, C. R. et al., 1997, Genes Dev. 11:824; Luo, C. et al., 1996, Proc. Natl. Acad. Sci. USA 93:8907), have been identified within the N-terminal domain.

5 The conserved serine residues in the SRR motif were found to be constitutively phosphorylated by cellular kinases and can be dephosphorylated by CN (Beals, C. R. et al., 1997, Genes Dev. 11:824). Deletion of CRI in human NF-ATx1 (hNF-ATx1) or mutation of serines in the SRR motif of NF-ATc led to the constitutive nuclear  
10 translocation of either hNF-ATx1 or NF-ATc (Masuda, E. S. et al., 1997, Mol. Cell. Biol. 17:2066; Beals, C. R. et al., 1997, Genes Dev. 11:824). Furthermore, at least two conserved NLSs have been reported to be essential for the nuclear translocation of NF-ATc; one NLS located in RSD is associated with the majority of  
15 phosphorylated serines in SRR (Beals, C. R. et al., 1997, Genes Dev. 11:824). Thus, NLS is probably masked by these phosphorylated serine residues. Both the domain interacting with CN and residues dephosphorylated by CN have been mapped within the N-terminus of NF-AT (Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15:2697; Beals, C. R. et al., 1997, Genes Dev. 11:824; Luo, C. et al., 1996, Proc. Natl. Acad. Sci. USA 93:8907), suggesting that the N-terminal domain of NF-AT is a target of CN action involved in major activities of the Ca<sup>2+</sup> signaling pathway and is important for the nuclear localization of NF-AT. However, detailed interaction between CN  
20 and NF-AT has not been clarified.

#### SUMMARY OF THE INVENTION

Structural and functional analyses of the N-terminal domain of murine NF-ATx1 (mNF-ATx1), (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157), a member of the NF-AT family, have defined two distinct  
30 CN binding regions (CNBRs), CNBR1 and CNBR2, which are located in the region preceding the SP boxes of serine/proline-rich sequences and the region between the SP boxes and Rel similarity domain,

respectively. Each of the two CN binding regions has the capacity to independently bind CN. The binding of mNF-ATx1 to CN was abolished by deletion of these two regions, yet was unaffected by the individual deletion. In contrast, the nuclear translocation of mNF-ATx1 was much reduced when only CNBR2 was removed. Luciferase assay revealed that both regions are required for mNF-ATx1-dependent activation of the murine IL-2 promoter. Most importantly, recombinant CNBR2 bound CN with a higher affinity, and when expressed in Jurkat cells, it functioned as a dominant negative mutant that prevented the transcription driven by exogenous mNF-ATx1, probably by interfering with the function of CN. The present invention revealed important features of the interaction of mNF-ATx1 with CN via the CN binding region, and light was shed on a structure-function model of mNF-ATx1 protein. The finding that one of two CN binding regions acts as an inhibitor of mNF-ATx1 opens the way for development of immunosuppressive agents. The present invention provides a new opportunity for pharmacological intervention with  $Ca^{2+}$ -dependent signaling events.

In one aspect, the invention relates to CN binding polypeptides and DNAs encoding them, and methods for their production.

Another aspect of the invention relates to methods for using polypeptides and polynucleotides of the invention. In particular, the present invention relates to a method for screening a compound that inhibits interaction between NF-AT and CN using the polypeptides of the present invention.

Still another aspect of the present invention is pharmaceutical compositions comprising a polypeptide of the present invention or a compound isolable by the above screening method. The pharmaceutical compositions can be used to inhibit interaction between NF-AT and CN. CN/NF-AT signal transduction is involved in induction of an immune response. Immunoreaction can thus be suppressed by inhibiting interaction between NF-AT and CN. The pharmaceutical compositions of this invention are especially



useful for suppressing rejection after transplantation of organs and treating or preventing autoimmune diseases. The polypeptides of this invention are useful for preventing hypercardia and hypertrophy of the vascular wall.

5 More specifically, the present invention relates to:

(1) a polypeptide having Calcineurin-binding activity selected from the group consisting of:

(a) polypeptides comprising the amino acid sequence set forth in SEQ ID NO: 1 (CNBR1: positions 25 to 142 of mNF-ATx1) or SEQ ID  
10 NO: 2 (CNBR2: positions 321 to 406 of mNF-ATx1);

(b) polypeptides corresponding to the polypeptides of (a) contained in NF-Atx family proteins;

(c) polypeptides of (a) or (b) in which one or more amino acids are added, deleted, substituted, and/or inserted; and

15 (d) fusion polypeptides comprising a polypeptide of (a), (b) or (c) and one or more other polypeptides;

(2) a DNA encoding the polypeptide of (1);

(3) a vector comprising the DNA of (2);

(4) a transformant carrying the DNA of (2) or the vector of (3);

20 (5) a method for producing the polypeptide of (1), the method comprising culturing the transformant of (4), and recovering the expressed polypeptide from the transformant or the culture supernatant;

(6) a method for screening a compound that inhibits the  
25 interaction between Calcineurin and NF-AT, the method comprising:

(a) contacting the polypeptide of (1) with Calcineurin in the presence or absence of a sample;

(b) detecting the binding activity of the polypeptide to Calcineurin; and

30 (c) selecting a compound that reduces the binding activity compared to the binding activity detected in the absence of a sample;

(7) a compound isolable by the screening method of (6);

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(8) a pharmaceutical composition comprising the compound of (7) as an active ingredient;

(9) a pharmaceutical composition comprising the polypeptide of (1) as an active ingredient;

5 (10) a method of suppressing immune, the method comprising administering the pharmaceutical composition of (8) to a patient in need of immunosuppression; and

(11) a method of suppressing immune, the method comprising administering the pharmaceutical composition of (9) to a patient  
10 in need of immunosuppression.

(12) a method of preventing the hypertrophy of cardiac smooth muscle or vascular smooth muscle, the method comprising administering the pharmaceutical composition of (8) to a patient.

(13) a method of preventing the hypertrophy of cardiac smooth  
15 muscle or vascular smooth muscle, the method comprising administering the pharmaceutical composition of (9) to a patient.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 Fig. 1 shows that CN interacts with the N-terminal domain of mNF-ATx1. Equal amounts of cell lysates isolated from pBJ5-CNA and pBJ5-CNB transfected COS-7 cells were incubated in the presence of glutathione-Sepharose 4B-bound GST-XND fusion protein (lane 3), glutathione-Sepharose 4B-bound GST alone (lane 4), and glutathione-Sepharose 4B alone (lane 5). Following binding and  
25 washing, the bound fraction was eluted and analyzed by SDS-PAGE and examined by Western blotting using either anti-CNA (upper panel) or anti-GST Ab (lower panel). In lanes 1 and 2, purified GST-XND fusion protein and the cell lysate isolated from CN-transfected COS-7 cells were subjected to SDS-PAGE directly for  
30 Western blotting assay, as the controls. Size markers are shown in kilodaltons on the left.

Fig. 2 schematically shows deletion mutants of GST-mNF-ATx1 fusion protein. Numbers indicate amino acid positions in mNF-ATx1 (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157) of different  
35 mutants. The SP boxes and the RSD are indicated.

Fig. 3 shows the CN binding activities of different mNF-ATx1 GST fusion proteins. Equal amounts of COS-7 cell lysates that had been transfected with pBJ5-CNA and pBJ5-CNB were incubated with glutathione-Sepharose 4B-bound GST fusion proteins of XND (lane

Fig. 4 schematically shows the N-terminal deletion mutants of mNF-ATx1. mNF-ATx1 $\Delta$ R1 and mNF-ATx1 $\Delta$ R2 were prepared by deleting R1 and R2, respectively, in the N-terminal domain of mNF-ATx1, as described in EXAMPLES. The SP boxes, NLSs, and RSD are indicated.

Fig. 6 shows effects of deletion mutations of mNF-ATx1 on transactivation of the IL-2 promoter. COS-7 cells were cotransfected with pmoIL-2-321Luc and pBJ5-CNA plus pBJ5-CNB along with the indicated expression plasmids. The transfected cells either were not stimulated or were stimulated for 8 h with PMA/A23187 as described in EXAMPLES. The relative luciferase unit (RLU) was normalized to the protein concentration by the bicinchoninic acid Protein Assay Reagent (Pierce, Rockford, IL). Data shown here are data derived from three independent transfection experiments.

Fig. 8 shows that recombinant CNBR2 suppresses mNF-ATx1-mediated transcription activity. Jurkat cells were transfected with pNF-AT72Luc reporter and pCMV-SEAP with pME-18S or pME-mNF-ATx1

alone or together with pcDNA-His-CNBR2. The empty expression vector pcDNA3.1/His was used to adjust the total amount of DNA transfected in each transfection, as required, and was used as a control. The transfected cells were either unstimulated or stimulated with PMA/A23187 for 8 h. In all transfections, pCMV/SEAP was included to monitor transfection efficiency. Luciferase activity values, given in relative luciferase units (RLU), were normalized to protein amounts in the lysates and to transfection efficiency. The data shown here were derived from three independent transfection experiments.

Fig. 9 shows the functional structure of the N-terminal domain of mNF-ATx1. The mNF-ATx1 protein contains two distinct CNBRs (CNBR1 and CNBR2) and other potential motifs that show the sequence conservation to hNF-ATx1 and other family members. C/CM2 of NF-AT4/NF-AT1 (Aramburu, J. et al., 1998, Mol. Cell 1:627; Zhu, J. et al., 1998, Cell 93:851) shown as indicated, overlaps with CNBR1 of mNF-ATx1. The functions of these motifs are discussed below.

Fig. 10 shows the alignment of amino acid sequences in N-terminal domains of NF-ATx polypeptide family. mNFATx; mouse NFATx [Ac. No. BAA12833 (D85612)], hNFATx; human NFATx [Ac. No. AAC27434 (AC004531), AAB46597 (U85430), AAB46596 (U85429), AAB46595 (U85428), AAA86308 (U14150)], NFATc3; human and mouse NFATc3 [pir: locus A57410 (Ho, S.N. et al., 1995, J. Biol. Chem. 270 (34), 19898-19907), AAA93249 (U28807), NP\_004546 (NM\_004555)].

Fig. 11 shows the alignment of amino acid sequences in N-terminal domains of human members of the NFAT polypeptide family. Sequences for NFAT1 (Luo, C.E. et al., 1996, Mol. Cell. Biol. 16: 3955-66), NFATc (Northrop, J.P. et al., 1994, Nature 369:497-502), NFATx (Hoey, T. et al., 1995, Immunity 2: 461-72; Masuda, E.S. et al., 1995, Mol. Cell. Biol. 15: 2697-706), and NFAT3 (Hoey, T. et al., 1995, Immunity 2: 461-72) were aligned by using the Lasergene Megalign program (DNASTar, Madison, Wis.), with additional

adjustments. Residues are numbered on the right and end at sequences corresponding to the start of their RSDs. Consensus residues, shown above the alignment, are a 100% match for the residue group. Residue groupings were as follows: DE; HKR; AGILV; 5 NQ; FWY; ST; P; CM. Residues that match the consensus are shaded (with solid black).

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is described below. All the patents, 10 publications, and literature references described herein are incorporated by reference.

Ca<sup>2+</sup> signal- and NF-AT-dependent transactivation is a complex process. NF-AT rapidly translocates into the nucleus upon Ca<sup>2+</sup> signaling, an event prevented by CsA and FK506 and hence requiring 15 the action of CN. Association between CN and NF-AT1/NF-ATx/4 has been reported (Masuda, E. S. et al., 1997, Mol. Cell. Biol. 17:2066; Luo, C. et al., 1996, Proc. Natl. Acad. Sci. USA 93:8907; Aramburu, J. et al., 1998, Mol. Cell 1:627). Present invention describes identification and characterization of the CN binding regions in 20 NF-AT. Using a series of truncated mNF-ATx1 proteins carrying a different N-terminal portion, the inventors identified two distinct CN binding regions responsible for mNF-ATx1 interacting with CN. Removal of either region alone did not abolish CN binding of mNF-ATx1, but deletion of both regions did so (Fig. 3). These 25 results are consistent with the previous data that multiple CN binding sites are present in the N-terminal domain of human NF-ATx1 (hNF-ATx1) and contribute to hNF-ATx1-CN interactions (Masuda, E. S. et al., 1997, Mol. Cell. Biol. 17:2066).

Like other NF-AT family members, the N-terminal domain of 30 mNF-ATx1 is rich in serine/proline residues and appears to play an important role in controlling the subcellular localization of NF-AT (Rao, A. et al., 1997, Annu. Rev. Immunol. 15:707; Masuda, E. S. et al., 1999, Cell. Signaling 10:599). As demonstrated in

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the CN binding assay, GST-XND fusion protein that had been incubated with the cell lysates of CNA/B-transfected COS cells migrated slightly slower than in the absence of the cell lysates (Fig. 1, compare lane 1 with lane 3). It is likely that the change in mobility is due to a phosphorylation of this protein by kinase(s) existing in COS-7 cells.

Among the CNBRs of mNF-ATx1 identified herein, CNBR2 (extending 86 amino acid residues located between the SP boxes and the RSD of mNF-ATx1) has unique properties. First, the CNBR2 fusion protein showed a strong binding activity to CN. Second, only when CNBR2 was removed from mNF-ATx1 was the nuclear translocation of mNF-ATx1 severely impaired (Figs. 4 and 5), even although CNBR1 was present. Most recently, the CN binding site of C/CM2 sequence was mapped within the corresponding region of CNBR1 in NF-AT1 and NF-AT4 molecules, respectively (Fig. 9) (Aramburu, J. et al., 1998, Mol. Cell 1:627; Zhu, J. et al., 1998, Cell 93:851). The sequence of this putative CN binding site was also noted and conserved in CNBR1 of mNF-ATx1 protein, suggesting that CNBR1 might be commonly used among the different NF-AT family members for CN interaction. In contrast to the present invention, their results showed a constitutively cytoplasmic localization of NF-AT4 and NF-AT1 when this CN binding site was deleted. In one assay system described herein, CNA and CNB were overexpressed during cotransfection with mNF-ATx1 deletion mutants, thus possibly overcoming the requirement of CNBR1 for mNF-ATx1 and transport mNF-ATx1 $\Delta$ R1 to the nucleus through interaction with CNBR2. Likewise, Zhu et al. found that when coexpressed with CN, the NF-AT4 mutant, in which the C sequence (putative CN binding site) was deleted, translocated into the nucleus upon activation of Ca<sup>2+</sup> signaling pathway (Zhu, J. et al., 1998, Cell 93:851). It is noteworthy that although the nuclear translocation of mNF-ATx1 was impaired dramatically by deleting CNBR2 (Fig. 5), it was not blocked completely; the mNF-ATx1 $\Delta$ R2 molecule was present in the nucleus of approximately

10% of transfected cells upon activation (data not shown). It appears that CNBR1 may play a lesser role in mediating mNF-ATx1 nuclear translocation; the amount of mNF-ATx1 translocated with two CN contact points at both CNBR1 and CNBR2 may be greater than the amount elicited by single contact point at CNBR2. The requirement of CNBR2 for the nuclear translocation of mNF-ATx1 may mean that CNBR2 is an essential element for transducing CN-triggered signaling on mNF-ATx1. This idea is supported by the finding that when expressed in Jurkat cells, recombinant CNBR2 suppressed the transcriptional enhancing activity of wild-type mNF-ATx1 (Fig. 8). Compared with CNBR2, it seemed likely that expressed CNBR1 did so to a lesser extent under the same conditions as well as under the conditions in which different amounts of transfected CNBR1 were used (data not shown). Moreover, the level of inhibition of CNBR2 was comparable to that of the whole N-terminal portion of the hNF-ATx molecule.

The mechanism of determination of the intracellular localization of the NF-AT family is a subject of considerable interest. It seems likely that phosphorylation/dephosphorylation of NF-AT is important for determining intracellular localization; NF-AT resides in the cytoplasm of resting cells in a phosphorylated state (Shibasaki, F. et al., 1996, Nature 382:370; Luo, C. et al., 1996, J. Exp. Med. 184:141; Timmerman, L. A. et al., 1996, Nature 383:837). Upon cell activation, CN dephosphorylates NF-AT directly and, in turn, induces NF-AT nuclear translocation. In this regard, CNBR2 forming a complex with CN probably makes CN accessible to phosphorylated residues, thereby inducing dephosphorylation of these residues, an event essential for the nuclear translocation of NF-AT.

It has been reported that an inhibitory sequence of 60 amino acid residues, termed CRI sequence, is located in the region preceding the SP boxes of hNF-ATx1 (Fig. 9). The deletion of this CRI sequence leads to nuclear translocation of hNF-ATx independent of  $\text{Ca}^{2+}$

signaling (Masuda, E. S. et al., 1997, Mol. Cell. Biol. 17:2066). Likewise, Beals et al. mapped an SRR motif in hNF-ATc with 23 amino acids located within the corresponding CRI region of hNF-ATx (Fig. 9). Mutation of serines in the SRR motif results in nuclear localization of NF-ATc (Beals, C. R. et al., 1997, Genes Dev. 11:824). Therefore, it is reasonable to speculate that the NLS(s) is masked by phosphorylated serine residues in CRI/SRR (Beals, C. R. et al., 1997, Genes Dev. 11:824). mNF-ATx1ΔR1, in which the deletion extends to the CRI/SRR, translocated to the nucleus in a stimulation-dependent manner.

Transcriptional activation of IL-2 promoter mediated by mNF-ATx1 increased markedly in PMA/A23187-stimulated COS-7 cells, when coexpressed with CNA and CNB. However, the transcriptional activity of mNF-ATx1 was reduced after either R1 or R2 was removed from mNF-ATx1 (Fig. 6). The reduction in transactivation activity of mNF-ATx1ΔR1 is probably due to the lack of the N-terminal transactivation domain (TAD) by deletion of the R1 region. Detailed analysis of the N-terminal TAD has been reported in the case of NF-AT1, in which the TAD was mapped within the first 100 amino acids (Luo, C. et al., 1996, J. Exp. Med. 184:141). In hNF-ATx, TAD is localized within the first N-terminal 400 amino acids. Alternatively, the R1 binding site for CN has no functional significance. The remaining transactivation activity of mNF-ATx1ΔR1 is probably stimulated by translocated mNF-ATx1ΔR1, together with another TAD, which has been mapped at the C-terminus of hNF-ATx1 sharing sequence conservation with mNF-ATx1 (Imamura, R. et al., 1998, J. Immunol. 161:3455). Similarly, PMA/A23187-induced transcription activity of mNF-ATx1 lacking R2 was reduced; however, this was due to impaired nuclear entry (Figs. 5 and 6), although a low level of translocated molecules may have contributed to the activity to some extent. In fact, when both of two CNBRs were deleted, PMA/A23187-induced transcription activity of mNF-ATx1 was abolished, indicating that full



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9: 295-304; Ranger, A. M. et al., 2000, J. Exp. Med. 191: 9-22; Ranger, A. M. et al., 1998, Immunity 9: 627-35; de la Pompa, J. L. et al., 1998, Nature 392: 182-6). As described above, since CNBR2 is expected to specifically inhibit the activation of NF-ATx family, but not the activation of NF-AT1, etc., the role of NF-ATx can be examined by specifically inhibiting NF-ATx without affecting NF-AT1, etc. using CNBR2 polypeptide under particular circumstances (for example, T cell, helper T cell subsets *in vivo*, etc.). Considering that CNBR2 polypeptide can exclusively inhibit NF-ATx without affecting the NF-AT1 activity, the polypeptide can be a substitute of CsA as an immunosuppressant with little side effect.

As described below, NF-AT3 has been particularly known of its activity to cause hypertrophy of smooth muscle. Since the CNBR2 regions in NF-AT3 and NF-AF are relatively well conserved, interaction between NF-AT3 and CN may be inhibited by CNBR2 polypeptide. Therefore, CNBR2 expectedly inhibits hypercardia and hypertrophy of vascular smooth muscle associated with hypertension.

## Abbreviations

Abbreviations used herein: CN, Calcineurin; CNA and CNB, subunits of wild-type Calcineurin; CsA, cyclosporin A; NF-AT, nuclear factor of activated T cells; AP1, activating protein-1; RSD, Rel similarity domain; NLS, nuclear localization signal; CRI, Calcineurin-regulated inhibitory; SRR, serine-rich region; h, human; m, murine; GST, glutathione S-transferase; CNBR, Calcineurin-binding region; TAD, transactivation domain; NES, nuclear export signal.

## Definitions

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"NF-AT polypeptide" used herein means a general name of NF-AT family proteins. Nuclear factor of activated T-cells (NF-AT) was identified as a protein involved in the expression of cytokine IL-2. Cloning and characterization of NF-AT gene are described in Masuda, E.S. et al., 1995, Mol. Cell. Biol. 15: 2697-706; Ho, S.N. et al., 1995, J. Biol. Chem. 270: 19898-907; Hoey, T. et al., 1995, Immunity 2: 461-72; Luo, C.E. et al., 1996, Mol. Cell. Biol. 16: 3955-66; Northrop, J.P. et al., 1994, Nature 369: 497-502. Five members of NF-AT family proteins are known, and nomenclature of them is shown in the table below.

Table 1

Current Approved Symbol	Another symbol	Literature Aliases	Sequence accessions	Reference PMID
NFATC1	NFAT2	NFAT2, NF-ATC, NFATc	U08015	9506523, 8202141
NFATC2	NFAT1	NFAT1, NF-ATP, NFATp	U43341/2	7842733, 8668213
NFATC3	NFAT4	NFAT4, NFATX	L41067, U14510	7749981, 7650004, 7739550
NFATC4	NFAT3	NFAT3	L41066	7749981
NFATC5				
NFAT5	NFAT5	TonE-BP, KIAA0827	AF089824 AB020634 AF134870	10051678 10377394

NF-AT polypeptide is usually a Rel protein comprising the Rel similarity domain, and its activation is regulated by calcium. Nuclear transport of this polypeptide is generally inhibited by cyclosporin A (CsA), resulting in the inhibition of its activation. NF-AT polypeptide used in this invention includes not only wild-type NF-AT polypeptides but also fragments thereof, modified NF-AT polypeptides, analogues thereof, etc.

"NF-ATx polypeptide" used herein means one of the NF-AT family members, an NF-AT polypeptide which is highly expressed in thymus (Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15: 2697-706; Liu,

J. et al., 1997, Mol. Biol. Cell. 8: 157). The NF-ATx polypeptide includes NF-AT4 polypeptide and NF-ATc3 polypeptide.

"NF-ATx1 polypeptide" used herein means one isoform among NF-ATx. "mNF-ATx1 polypeptide" means a mouse NF-ATx1 polypeptide, and is encoded by the mNF-ATx1 cDNA set forth in Accession Number D85612 (Liu, J. et al., 1997, Mol. Biol. Cell. 8: 157). "hNF-ATx1 polypeptide" means a human NF-ATx1 polypeptide, and is encoded by the hNF-ATx1 cDNA set forth in Accession Number U14510 (Masuda, E. S. et al., 1995, Mol. Cell, Biol. 15: 2697-706).

"Calcineurin (CN)" is generally known as a calcium/calmodulin-dependent serine/threonine phosphatase, existing as a heterodimeric protein composed of a calmodulin-binding catalytic subunit (CNA) and a  $\text{Ca}^{2+}$ -binding regulatory subunit (CNB) (Clipstone, N. A. and Crabtree, G. R., 1992, Nature 357 (6380): 695-7; Tsuboi, A. et al., 1994, Mol. Cell. Biol. 5: 119-28). CN is a target of CsA and FK506, regulating the activation of NF-AT. In this invention Calcineurin (CN) includes not only a heterodimeric protein but also CNA alone or CNB alone and derivatives thereof.

"Polypeptides" used herein mean peptides or proteins comprising two or more amino acid residues linked by peptide bond or modified peptide bond. Polypeptides may include peptide isosteres. Polypeptides usually include short-chain molecules such as those known as peptides, oligopeptides, or oligomers, and also long-chain molecules known as proteins. Polypeptides may be spontaneously modified by post-translational modification, or artificially modified in their peptide backbone, amino acid side chain, amino or carboxyl termini, etc. Polypeptides may be branched by ubiquitination, etc. or cyclized. Examples of modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of

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phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, hydroxylation, iodination, methylation, myristoylation, oxidation, phosphorylation, ubiquitination, etc.

### Polypeptides of the Invention

Polypeptides of this invention include a polypeptide set forth in SEQ ID NO: 1 or SEQ ID NO: 2. Both of these polypeptides have the binding activity to Calcineurin (CN). Polypeptides comprising amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 in which one or more amino acids are added, deleted, substituted, and/or inserted are also included in the polypeptides of this invention as far as they maintain the binding activity to CN. Such polypeptides include, for example, a partial polypeptide derived from mNF-ATx1 which shares an overlapping region with SEQ ID NO: 1 or SEQ ID NO: 2 but is not identical with these sequence. For example, SEQ ID NO: 1 is a partial polypeptide consisting of amino acids 25 to 143 of mNF-ATx1 (Ac. No. D85612; Liu, J. et al., 1997, Mol. Biol. Cell. 8: 157), but the same polypeptides whose amino terminus is not position 25 but the other position on the amino or carboxyl terminal side from position 25 is included in the polypeptide of this invention, and the polypeptides whose carboxyl terminus is not exactly at position 143 likewise. SEQ ID NO: 2 is a partial polypeptide comprising amino acids 321 to 406 of mNF-ATx1. The polypeptides of this invention include the same polypeptides whose amino or carboxyl termini is not positions 321 or 406 but at other positions on the amino or carboxyl terminal side from positions 321 or 406. Thus, amino and carboxyl termini can be any positions in the amino acid sequence of mNF-ATx1.

Polypeptides of this invention also include partial polypeptides of the polypeptide encoded by human NF-ATx1 gene (Ac. No. U14510; Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15: 2697-706),

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comprising regions corresponding to the above-described polypeptides. A polypeptide in a region of a protein "corresponding to" a polypeptide in a region of another protein means a polypeptide at the region shared by both proteins found when both amino acid sequences are aligned. Alignment of amino acid sequences can be performed by Clastal W Alignment using, for example, a "Mac Vector" software (Oxford Molecular). Alignment of N-terminal amino acid sequences of NF-AT family polypeptides are shown in Figs. 10 and 11 (Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15: 2697). More specifically, in the human NF-ATx1 polypeptide (Ac. No. AAA86308), for example, the regions corresponding to CNBR1 (SEQ ID NO: 1) and CNBR2 (SEQ ID NO: 2) of mNF-ATx1 are amino acids 25 to 143 and 321 to 406, respectively. These amino acid sequences are shown in SEQ ID NOS: 3 and 4. Polypeptides of this invention may be derived from regions corresponding to the above-described polypeptides in other NF-AT family proteins.

Polypeptides of this invention can be isolated using hybridization techniques or gene amplification techniques. It is a routine for those skilled in the art to obtain DNA encoding a polypeptide of this invention from DNAs highly homologous to the DNA sequence encoding the mNF-ATx1 protein or a portion thereof isolated from DNA samples derived from organisms of the same or different species using hybridization techniques (Ausubel, F. M. et al., Eds. (1992) Current Protocols in Molecular Biology, 2.9, Green Publishing Associates and Wiley-Interscience, JOHN WILEY & SONS, NY). Conditions for hybridization can be suitably determined. Thus, it is possible to isolate DNA encoding the mNF-ATx1 protein or its structural analogues by hybridization and determine the region of polypeptides of this invention in the isolated DNA. Animals used for isolating such proteins include, for example, rabbits, chicken, pigs, cattle, etc. besides primates such as humans and monkeys, and rodents such as rats and mice, but

are not limited thereto. The region corresponding to polypeptides of this invention in proteins other than mNF-ATx1 protein can be selected by aligning amino acid sequences of other proteins with that of mNF-ATx1 protein and determining the regions in the amino acid sequences corresponding to a polypeptide of this invention that is the partial polypeptide of mNF-ATx1 protein. Once the region is determined, a recombinant protein can be prepared by appropriately inserting the DNA region encoding the partial polypeptide into an expression vector.

It is also possible to prepare polypeptides of this invention by amplifying DNAs encoding mNF-ATx1 protein, its structural analogues, or portions thereof using polymerase chain reaction (PCR) (Ausubel, F. M. et al., Eds. (1992) Current Protocols in Molecular Biology, 15, Green Publishing Associates and Wiley-Interscience, JOHN WILEY & SONS, NY).

The amino acid sequences of polypeptides of this invention including partial polypeptides of wild-type mNF-ATx1 proteins, may have mutations as long as they have the binding activity to CN. Such mutations may be introduced spontaneously or artificially. In the case of artificial amino acid substitution, the activity of the intact polypeptide can be maintained by substituting amino acid(s) with the one(s) of similar property. Polypeptides of this invention include the partial polypeptides of the wild-type protein modified by the conservative amino acid substitution.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with another residue having a chemically similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline,

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phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). It is also possible to isolate a polypeptide with a stronger binding activity to CN by varying amino acids of polypeptides at random and screening those variants.

In polypeptides of this invention with mutations in amino acid sequence of the partial polypeptide of the wild-type protein (e.g. SEQ ID NO: 1 or SEQ ID NO: 2), the number of amino acids that can be added, deleted, substituted, and/or inserted is usually 50 or less, preferably 30 or less, more preferably 15 or less, and still more preferably 10 or less.

A polypeptide of this invention may be a fusion polypeptide with other polypeptide species. A "fusion polypeptide with other polypeptide species" used herein means a polypeptide produced by linking at least two polypeptides that are not joined in nature, and can be produced by expressing a nucleic acid comprising the coding regions of the polypeptides linked so as to be in frame. Other polypeptide species includes tag sequences, GFP, maltose-binding protein, glutathione S-transferase (GST), etc., but are not limited thereto.

The binding activity to CN can be assessed by known techniques such as pull-down assay, immunoprecipitation, ELISA, Two hybrid system, BIACORE, etc. Although the wild-type CN is composed of plural subunits (subunits A and B), for example, CNA or ACNA lacking the regulatory region (constitutive active CNA) can be used in the binding assay to NF-AT. However, since the co-existence of CNB with CNA confers a high binding capacity on CN, both subunits of CN are preferably used in the assay. CN used in the assay can be either the one expressed in *Escherichia coli* or the one purified or partially purified from tissues abundant in CN such as brain, etc.

For example, a desired polypeptide is expressed as a fusion

polypeptide with GST. Cell lysates which express CN (CNA and CNB) are incubated with glutathione-Sepharose 4B-bound GST-fusion protein in buffer containing 150 mM NaCl, 50 mM HEPES buffer, 10  $\mu$ M CaCl<sub>2</sub>, 0.25% Nonidet P-40, and protease inhibitors. After washing the beads, the glutathione-Sepharose 4B-bound fraction is eluted by boiling in gel loading buffer and analyzed by SDS-PAGE. Bound proteins can be visualized using anti-CNA and anti-GST mAbs.

By these assays, it is also possible to isolate a polypeptide of minimum length retaining CN-binding capability and a mutant polypeptide with a stronger CN-binding activity.

Polypeptides of this invention can be prepared by purifying the wild-type polypeptide, or prepared as recombinant polypeptides using recombinant technology well known to those skilled in the art. The polypeptides may also be synthesized. A recombinant polypeptide can be prepared, as described below, for example, by transfecting suitable host cells with a vector into which DNA encoding the polypeptide of this invention is inserted and recovering the polypeptide expressed in the transformant.

#### **DNAs of the Invention**

The present invention also relates to DNAs encoding polypeptides of this invention. There is no particular limitation in the type of these DNAs as long as they can encode polypeptides of this invention, including cDNA, genomic DNA, chemically synthesized DNA, etc. DNAs comprising nucleotide sequences based on the degeneracy of genetic code are also included as long as they can encode polypeptides of this invention. DNAs set forth in SEQ ID NO: 1 or 2 can be isolated by, for example, standard methods such as hybridization using the DNA sequence encoding mNE-ATx1 protein (Ac. No. D85612; Liu, J. et al., 1997, Mol. Biol. Cell. 8: 157) or a portion thereof as the probe, PCR using primers synthesized based on these DNA sequences, etc. These DNAs can also be synthesized with a DNA synthesizer.

DNAs of this invention can be used to produce polypeptides of this invention as recombinant proteins by inserting the DNA into the vector as described below, and introducing the vector into host cells. As described above, a fusion polypeptide with other polypeptide species can be produced by connecting the coding region of the polypeptide of this invention with that of the other polypeptide species so as to be in frame. Other polypeptide species include leader sequence, secretion signal, and sequence of pre- or pro-sequence. Addition of tag sequence can facilitate the purification of polypeptides. Examples of the tag sequence include 6 x His, HA tag, etc. DNAs of this invention can be modified so that they can encode fusion proteins with other proteins such as GFP, maltose-binding protein, glutathione *S*-transferase (GST), etc. DNAs of this invention may comprise, in addition to coding regions, non-coding sequences (non-transcriptional sequence, non-translational sequence, splicing sequence, poly A addition sequence, IRES, mRNA stabilization/destabilization sequence, etc.).

## **Vectors, Transformants, Expression**

The present invention also provides vectors carrying DNAs of this invention. There is no particular limitation in the type of vectors of this invention as long as they can stably retain inserted DNAs. Vectors of this invention include plasmids, phagemids, phages, cosmids, chromosomes, viruses, etc. The type of host cells is not particularly limited, and includes, for example, *Escherichia coli*, yeasts, plant cells, animal cells, etc. Preferable animal cells are, for example, insect cells and mammalian cells. Individuals of animals and plants can also be used as hosts.

Polypeptides of this invention can be prepared in a large scale, by, for example, using the *Escherichia coli* expression system. The polypeptides can be expressed using well-known expression vectors

such as pGMEX (Promega), etc. The expressed polypeptides can be secreted into the endoplasmic reticulum, periplasm, or outside of cells if secretion signal is suitably attached to the polypeptides. The extracellularly secreted polypeptides can be purified from the culture medium of the transformants. When the polypeptides are intracellularly expressed, the transformants are collected and lysed to recover the polypeptides. The polypeptides can be prepared by known protein purification methods such as ammonium sulfate precipitation, cationic or anionic exchange chromatography, gel filtration, affinity chromatography, HPLC, etc. Vectors can be introduced into host cells by the calcium chloride method, electroporation method, etc.

Vectors to be used for transfection of mammalian cells, include, for example, pME18s vector, etc. Cell lines into which the vectors are introduced include, for example, COS cells, CHO cells, 3T3 cells, BHK cells, 293 cells, etc. Host cells may not be necessarily the established cell line. For example, primary cultures, *Xenopus laevis* oocytes, etc. may be used. Vectors can be introduced into host cells by the calcium phosphate method, DEAE-dextran method, electroporation method, lipofection method, injection method, etc. (Ausubel, F. M. et al., Eds. (1992) Current Protocols in Molecular Biology, New York: Green Publishing Associates and Wiley-Interscience).

The vectors of this invention to be used for gene therapy are preferably virus vectors. The virus vectors include, for example, known vectors such as retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, etc. (Kurata, H. (1999) Immunity 11: 677-88; Robbins, P. D. (1998) Trends Biotechnol. 16: 35-40). For gene therapy, for example, the DNA of this invention is inserted into the vector, and the vector is administered to living bodies. Vectors may be administered either *in vivo* or *ex vivo*.

### Screening Assays

This invention also provides a method for screening a compound that inhibits the interaction between CN and NF-AT using the polypeptide of this invention. CN dephosphorylates NF-AT to  
5 convert it into an active form and induces its transport into nuclei. Therefore, the inhibition of interaction between CN and NF-AT will block signal transduction from CN to NF-AT.

The screening method of the present invention comprises: (a) contacting the polypeptide of this invention with Calcineurin in  
10 the presence or absence of a sample; (b) detecting the binding activity of the polypeptide to Calcineurin; and (c) selecting a compound that reduces the binding activity compared to the binding activity detected in the absence of a sample.

The sample to be used in the above method includes, for example,  
15 supernatants from culture medium of microorganisms, natural ingredients derived from plants or marine organisms, biological tissue extracts, cell extracts, expression products of a gene library, synthetic low molecular weight compounds, synthetic peptides, natural compounds, etc., but are not limited thereto. Peptides that strongly inhibit the binding between the polypeptide  
20 of this invention and CN can be isolated by, for example, screening a random peptide library.

The polypeptide of this invention as it is or a mutant polypeptide thereof can be used as the sample. As shown in Fig. 8, for example,  
25 the CNBR2 polypeptide of mNF-ATx1 expresses by itself a dominant negative phenotype against the binding of NF-AT to CN. Therefore, a minimum polypeptide unit in the amino acid sequence of CNBR2 polypeptide necessary for inhibiting the binding of NF-AT to CN can be identified by the screening assay of this invention using  
30 a partial peptide of the CNBR2 polypeptide as a sample taking the binding inhibition between the polypeptide of this invention and CN as an index.

The polypeptide of this invention used for screening may be, for

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example, in the form expressed on the cell surface, a cell membrane fraction of the cells, or the form bound to a carrier. CN to be used is, for example, CNA or ACNA lacking the regulatory domain (constitutive active CNA). Herein, the co-existence of CNB and  
5 CNA is preferable because it enhances the binding capacity to the polypeptides of this invention. CN can be the one expressed in *Escherichia coli* or the one purified or partially purified from tissues abundant in CN such as brain, etc.

The binding activity of the polypeptides of this invention to  
10 CN can be assessed, as described above, by well-known methods such as pull-down assay, immunoprecipitation, ELISA, Two hybrid system, BIACORE, etc.

More specifically, CNA or ACNA expressed in *Escherichia coli*, etc. is adsorbed onto a plastic support (for example, 96-well plate, etc.). In this case, CNA is preferably used together with CNB.  
15 In the presence of a test compound, a compound that inhibits the interaction between CN and the polypeptide of this invention is selected by adding the biotinylated polypeptide of this invention to the reaction system, and detecting the polypeptide bound to CN  
20 using avidin-bound antibody or avidin-bound fluorescence reagent, etc.

A method utilizing surface plasmon resonance, a high through put screening method in combinatorial chemistry techniques, etc. can also be used.

25 Compounds capable of reducing the binding activity of the polypeptide of this invention to CN obtained by this screening method can be used to develop novel drugs. Herein, "reducing the binding activity of the polypeptide of this invention to CN" means  
30 reducing the binding activity of the polypeptide of this invention to CN by either directly or indirectly acting on the polypeptide of this invention or CN. Therefore, compounds isolated by this screening method include not only compounds that reduces the binding of the polypeptide of this invention to CN by directly

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acting on either one of them but also compounds that reduce the binding activity without directly interfering with their binding itself.

The polypeptides, DNAs, vectors of the present invention, and compounds isolable by the screening method of this invention are useful for inhibiting the interaction between CN and NF-AT. The interaction between CN and NF-AT is inhibited in cells by introducing one or more polypeptides of the present invention or compounds isolable by the screening method of this invention into the cells, or by expressing either the DNAs or vectors of this invention in the cells, thereby blocking signal transduction from CN.

#### **Pharmaceutical Compositions and Administration**

The polypeptides of this invention or compounds isolable by the screening method of the invention can be administered to patients, for example, in a dose pharmaceutically effective to sufficiently inhibit the interaction between CN and NF-AT. The polypeptides of this invention may be modified ones or their derivatives. Modification or derivatization of polypeptides can lead to the improvement of their stability or cell permeability *in vivo*. The polypeptides of this invention may be in the form of salts thereof. The polypeptides in these forms can be administered, for example, intravenously or orally. The polypeptides of this invention or compounds isolable by the screening method of the invention can be administered in suitable combinations with pharmaceutically acceptable carriers. In addition, the DNAs or vectors of this invention can be administered in an amount to express and produce the polypeptides sufficient to inhibit the interaction between CN and NF-AT. The DNAs of this invention can be incorporated into retrovirus vectors, adenovirus vectors, etc. and introduced into patient cells *in vivo* or *ex vivo*.

The polypeptides of this invention or compounds isolable by the

screening method of the invention are useful as novel immunosuppressants with less side effects than conventional drugs. In addition, NF-AT is reportedly involved in hypertrophy of smooth muscle and skeletal muscle (Boss, V. et al., 1998, J. Biol. Chem. 273: 19664-71; Abbott, K. L. et al., 1998, Mol. Biol. Cell. 9: 2905-16; Wang, X. G. et al., 1997, Mol. Pharmacol. 52: 781-7; Musaro, A. et al., 1999, Nature 400: 581-5; Semsarian, C. et al., 1999, Nature 400: 576-81; Molkentin, J. D. et al., 1998, Cell 93: 215-28; Izumo, S. and H. Aoki, 1998, Nat. Med. 4: 661-2). The fact indicates that hypercardia and hypertrophy of the vascular wall can be prevented by inhibiting the activation of NF-AT in smooth muscle and/or skeletal muscle.

When the polypeptides of this invention or compounds isolable by the screening method of the invention are used as the drug, these can be administered to patients either as they are or as pharmaceutical compositions formulated by known pharmaceutical methods. The polypeptides or the compounds may be formulated, for example, together with pharmaceutically acceptable carriers or media, such as sterilized water, physiological saline, dextrose, glycerol, ethanol, vegetable oil, emulsifier, suspending agent, etc. The pharmaceutical compositions of this invention may be in the form of an aqueous solution, tablets, capsules, troches, buccal preparations, elixirs, suspensions, syrups, etc. Contents of active ingredients may be appropriately determined. The compositions can be administered to patients in general by known methods such as intra-arterial injection, intravenous injection, intraperitoneal injection, subcutaneous injection, oral administration, etc. Administration can be performed systemically or locally. Doses may vary depending on properties of these polypeptides or compounds, body weight or age of patients, administration method, symptoms, etc., and can suitably be selected by those skilled in the art. Usual doses typically range from about 0.01 mg/kg to 1 g/kg, preferably about 0.05 mg/kg to



100 mg/kg, and more preferably about 0.1 mg/kg to 50 mg/kg per day in a single dose or several divided doses. When the polypeptides or the compounds is encoded by DNA, gene therapy may be performed by incorporating the DNA into a vector for gene therapy and administering the vector *ex vivo* or *in vivo*.

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. These examples are offered by way of example and not by way of limitation. Variations and alternate embodiments will be apparent to those of skill in the art.

#### Example 1

##### CN interacts independently with two distinct regions within the N-terminal domain of mNF-ATx1

To determine whether the N-terminal domain of mNF-ATx1 could mediate the association with CN, the construct encoding the fusion protein (GST-XND) of the N-terminal domain of mNF-ATx1 (amino acids 25-406) (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157) with GST was made and expressed in *E. coli*. The purified GST-XND fusion protein was used for *in vitro* CN binding assay. The expression plasmid used for mNF-ATx1 (pME-mNF-ATx1) was previously described (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157). pBJ5-CNA and pBJ5-CNB, which encode CNA and CNB subunits, were provided by N. A. Clipstone and G. Crabtree (Stanford University, Stanford, CA) (Clipstone, N.A. and Crabtree, G.R., 1992, Nature 357:695-7; Tsuboi, A. et al., 1994, Mol. Cell. Biol. 5:119-28) .

For the CN binding assay, the purified GST-XND fusion protein or GST protein immobilized on glutathione-Sepharose 4B beads, or glutathione-Sepharose 4B alone was incubated with cell lysates isolated from COS-7 cells that had been transfected with the expression vectors for the wild-type CN (pBJ5-CNA and pBJ5-CNB) in buffer containing 150 mM NaCl, 50 mM HEPES buffer, 10  $\mu$ M CaCl<sub>2</sub>,

0.25% Nonidet P-40, 1  $\mu$ g/ml of leupeptin, 1  $\mu$ g/ml of aprotinin, 10 mM NaF, 1 mM NaV<sub>3</sub>O<sub>4</sub>, and 10 mM Na pyrophosphate. After washing the beads, the glutathione-Sepharose 4B-bound fraction was eluted by boiling in Laemmli's sample buffer and analyzed by SDS-PAGE followed by Western blot analysis, using anti-CNA (Sigma) mAbs and anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA) mAbs, respectively. As shown in Fig. 1, CN was detected when GST-XND was used (lane 3); however, no CN was observed when glutathione-Sepharose 4B-bound GST or glutathione-Sepharose 4B alone was used under the same conditions (lanes 4 and 5), suggesting that CN binding depended on the presence of the N-terminal domain of mNF-ATx1.

To locate precisely the CN binding portion within the N-terminal domain of mNF-ATx1, a series of GST fusion proteins of mNF-ATx1 mutants having the N-terminal truncations was prepared (Fig. 2A). For a series of GST fusion constructs, DNA fragments derived from the N-terminal domain of mNF-ATx1 were inserted into either pGEX4T-1 or pGEX-5X-3, according to the reading frame (Pharmacia, Piscataway, NJ). All GST fusion proteins and GST protein expressed in *Escherichia coli* strain BL21DE3 were affinity purified according to the manufacturer's instructions (Pharmacia). The CN binding assay was performed under the same condition as described above. These fusions were analyzed by SDS-PAGE followed by either Coomassie brilliant blue staining (data not shown) or Western blots using an anti-GST Ab (Fig. 3).

XNAR2 was a deletion mutant lacking the region between the SP boxes and the RSD. Binding assay with GST-XNAR2 fusion protein and COS-expressed CN showed that such deletion had no significant effect on its CN binding activity, compared with that of GST-XND fusion protein (Fig. 3). Likewise, when expressed in *E. coli* as a GST fusion protein, XNAR1, in which the N-terminal region preceding the SP boxes was removed from XND, still bound to CN (Fig. 3). However, when further deletion was made in XNAR1 at its

C-terminus to yield XNAR12, the CN binding activity of GST-XNAR12 was drastically reduced. The lack of CN binding activity of GST-XNAR12 fusion protein was not due to a lower amount of the protein used. Blotting of the filter by anti-GST Ab revealed that the amount of GST-XNAR12 fusion protein, which was more than the amount of GST-XND or GST-XNAR2 fusion, was still comparable to that of GST-XNAR1 fusion protein (Fig. 3, lower panel).

Taken together, these results showed that both GST-XNAR1 and GST-XNAR2 fusion proteins are capable of interacting with CN and that the individual deletion of either R1 or R2 did not abolish the potential of mNF-ATx1 to bind CN. Since GST-XNAR12, which contains the overlapping region of both GST-XNAR1 and GST-XNAR2, failed to bind CN, mNF-ATx1 is likely to contain two CN binding regions (CNBRs): R1, localized at the region preceding the SP boxes, contains the amino acid residues 25-188; R2, corresponding to the region between the SP boxes and RSD of mNF-ATx1, contains the amino acid residues 317-406.

#### Example 2

##### Removal of the R2 region results in impairment of the nuclear translocation of mNF-ATx1

Since the CN binding of mNF-ATx1 seemed to be mediated via either R1 or R2, the inventors next asked whether the subcellular localization of mNF-ATx1 would be affected by deleting R1 and R2. To address this question, the inventors prepared expression constructs of pME-mNF-ATx1ΔR1 and pME-mNF-ATx1ΔR2, in which R1 and R2 were deleted, respectively (Fig. 4). pME-mNF-ATx1ΔR1, pME-mNF-ATx1ΔR2, and pME-mNF-ATx1ΔR1/R2 were obtained from pME-mNF-ATx1 (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157) by deleting the N-terminal region preceding the SP boxes of mNF-ATx1 (nucleotide positions between 79-571, R1), the region between the SP boxes and the RSD (nucleotide positions between 966-1227, R2), and the region covering both R1 and R2 of mNF-ATx1, respectively.

As reported previously, in COS-7 cells, nuclear translocation of overexpressed mNF-ATx1 molecule depends on coexpression of the wild-type CN followed by stimulation of the cells with calcium ionophore (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157).

5 Therefore, pBJ5-CNA and pBJ5-CNB were cotransfected with pME-mNF-ATx1ΔR1 or pME-mNF-ATx1ΔR2 into COS-7 cells. mNF-ATx1ΔR1 and mNF-ATx1ΔR2 expressed in COS-7 cells were visualized by immunofluorescence staining as previously described (Liu, J. et al., 1997, Mol. Biol. Cell. 8:157). An affinity-purified  
10 polyclonal Ab, APαDS, raised against a bacterially produced recombinant peptide of human NF-ATx (hNF-ATx) extending from amino acid residues 387-728 (Masuda, E. S. et al., 1995, Mol. Cell. Biol. 15:2697), was used to detect mNF-ATx1 and its mutants. This Ab recognizes the RSD of hNF-ATx1. The secondary Ab used was  
15 FITC-labeled goat anti-rabbit IgG (Zymed, South San Francisco, CA). The immunostaining revealed that mNF-ATx1ΔR1 was present predominantly in the cytoplasm of unstimulated cells (Fig. 5A). Following activation of the cells by A23187, mNF-ATx1ΔR1 translocated to the nucleus of most transfected cells (Fig. 5B).  
20 In marked contrast, mNF-ATx1ΔR2 showed no significant redistribution to the nucleus in response to the activation of CN in immunostaining (Fig. 5, C and D); mNF-ATx1ΔR2 remained in the cytoplasm of 90% of transfected cells. Although the accumulation of mNF-ATx1ΔR2 in the nucleus was observed in some activated cells,  
25 it represented only a small population among the transfected cells compared with that of mNF-ATx1ΔR1.

These results indicate that R2 is a domain actively involved in the nuclear localization of mNF-ATx1 and is indispensable for this process, whereas R1 elicits less drastic effects and cannot by  
30 itself transport mNF-ATx1 to the nucleus.

### Example 3

Deletion of either the R1 or R2 region abolishes mNF-ATx1-mediated

transcriptional activation of the murine IL-2 gene

On the basis of the observation that removal of R2 reduced the nuclear translocation activity of mNF-ATx1, the inventors then examined the roles of R1 and R2 in mNF-ATx1-dependent gene activation. COS-7 cells were grown in DMEM medium containing 10% FCS, 50 U/ml of penicillin, and 50 µg/ml streptomycin. Jurkat cell lines were cultured in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 50 µM 2-ME, 50 U/ml of penicillin, and 50 µg/ml streptomycin. Transfections into COS-7 and Jurkat cells were conducted as previously described (Masuda, E. S. et al., 1997, Mol. Cell. Biol. 17:2066; Liu, J. et al., 1997, Mol. Biol. Cell. 8:157). pNF-AT72Luc containing the luciferase reporter gene under control of three copies of the murine IL-2 distal NF-AT sites (-290 to -261) (Tsuruta, L. et al., 1995, J. Immunol. 154:5255) and pmoIL-2-321Luc containing the luciferase reporter gene under control of the IL-2 promoter that covers the position of -321 to +46 were constructed as previously described (Tsuruta, L. et al., 1995, J. Immunol. 154:5255). pCMV-SEAP (Tropix, Bedford, MA), used as an internal control to evaluate transfection efficiency, is an expression vector of secreted alkaline phosphatase.

For luciferase assays performed in COS-7 cells, 6 µg of pmoIL-2-321Luc reporter plasmid and 6 µg of pME-mNF-ATx1, pME-mNF-ATx1ΔR1, pME-mNF-ATx1ΔR2, or pME-mNF-ATx1ΔR1/R2 with pBJ5-CNA plus pBJ5-CNB plasmid were used. pME-18S was used to adjust the total amount of DNA transfected, as required. In competition experiments, Jurkat cells were transfected with 1 µg of pNF-AT72Luc plus 0.5 µg of pCMV-SEAP and either 0.25 µg of pME-mNF-ATx1 or pME-18S alone or together with 0.75 µg of pCDNA-His-CNBR2. The pCDNA3.1/His empty vector was used to adjust the total amount of DNA transfected, as required. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI).

As shown in Fig. 6, when expression vectors encoding the wild-type mNF-ATx1 (pME-mNF-ATx1) as well as CNA and CNB were transfected

into COS-7 cells, exposure of the cells to PMA/A23187 enhanced transcription activity of the murine IL-2 promoter 4-fold over that of untreated cells. However, when pME-mNF-ATx1ΔR1 or pME-mNF-ATx1ΔR2 was used instead of pME-mNF-ATx1, mNF-ATx1-dependent transcription activity of the exogenous IL-2 promoter was decreased 1.8- or 1.9-fold following stimulation of the cells by PMA/A23187, respectively. Further deletion of both R1 and R2 resulted in the impairment of mNF-ATx1 transcription activity. Thus, both R1 and R2 are apparently essential for mNF-ATx1-dependent IL-2 promoter transcriptional activity.

#### Example 4

##### CNBR2 of mNF-ATx1 exhibits potent CN binding activity

The results described above show that R1 and R2 have different roles in transmitting the CN-mediated signal to mNF-ATx1; i.e., removal of R2 abolished nuclear translocation of mNF-ATx1, but removal of R1 did not, although both are involved in the CN binding event. To clarify why they elucidate the different functions, the inventors next examined the direct interactions of CN with these two regions. CNBR1, including amino acid residues of 25-143, is 45 amino acids shorter than R1; CNBR2, containing amino acid residues of 321-406, covers a region similar to that of R2 (Fig. 2B). Constructs encoding the GST fusion protein of CNBR1 or CNBR2 were expressed in *E. coli*. The purified GST-CNBR1 and GST-CNBR2 proteins were used to perform the binding assay under the same conditions as those described above. As expected, both GST-CNBR1 and GST-CNBR2 fusion proteins bound CN (Fig. 7), while no CN was observed when GST alone was used (data not shown). Remarkably, GST-CNBR2 fusion showed a much stronger CN binding activity than did the GST-CNBR1 fusion protein. This result was further confirmed using different amounts of purified CN, showing that the CN binding of GST-CNBR1 could be enhanced by the addition of increased amounts of purified CN. Nevertheless, its binding

activity was weaker compared with that of GST-CNBR2 under the same conditions (data not shown). It suggested that the different functions of two CN binding regions might be due to different CN binding potentials.

5

#### Example 5

#### CNBR2 polypeptide exerts a dominant negative effect on mNF-ATx1-dependent transcription activity

Previous study has shown colocalization of CN with NF-AT4 in the  
 10 nucleus of cells from the U2OS cell line (Shibasaki, F. et al., 1996, Nature 382:370). The inventors also observed that CN migrates to the nucleus together with mNF-ATx, when coexpressed in COS-7 cells that had been stimulated with A23187 (data not shown). Based on the evidence of potent CN binding activity of CNBR2, the  
 15 inventors hypothesized that recombinant CNBR2 acts in a dominant negative manner by titrating out CN interacting with mNF-ATx1. To test this possibility, CNBR2 cDNA fragment derived from mNF-ATx1 was constructed into the His-tagged expression vector pcDNA3.1/His (Invitrogen, Carlsbad, CA) to yield pcDNA-His-CNBR2. As shown in  
 20 Fig. 8, when Jurkat cells were cotransfected with pNF-AT72Luc and pcDNA3.1/His empty vector, the promoter activity, which was very low in the cells before stimulation, increased following treatment of the cells with PMA/A23187. This activity is probably supported by the action of endogenous NF-AT. When pME-mNF-ATx1 was  
 25 introduced into the cells, the promoter activity was further enhanced as much as 2.9-fold following stimulation. Interestingly, this enhancement by mNF-ATx1 was suppressed when the cells were transfected with pME-mNF-ATx1 along with pcDNA-His-CNBR2. Interestingly, the inventors also found that expressed CNBR2  
 30 elicited an inhibitory effect on the reporter gene that was transactivated by the endogenous NF-AT; however, this effect was much less effective compared with that of mNF-ATx1-dependent reporter gene activity. Therefore, as predicted, CNBR2

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polypeptide, containing a CN binding region with high affinity but lacking the DNA binding domain and the transactivation domain, acts in a dominant negative manner in mNF-ATx1-dependent reactions.



What is claimed is:

1. A polypeptide having Calcineurin-binding activity selected from the group consisting of:

(a) polypeptides comprising the amino acid sequence set forth in  
5 SEQ ID NO: 1 or SEQ ID NO: 2;

(b) polypeptides corresponding to the polypeptides of (a) contained in NF-Atx family proteins;

(c) polypeptides of (a) or (b) in which one or more amino acids are added, deleted, substituted, and/or inserted; and

10 (d) fusion polypeptides comprising a polypeptide of (a), (b) or (c) and one or more other polypeptides.

2. A DNA encoding the polypeptide of claim 1.

3. A vector comprising the DNA of claim 2.

15 4. A transformant carrying the DNA of claim 2 or the vector of claim 3.

5. A method for producing the polypeptide of claim 1, the method comprising culturing the transformant of claim 4, and recovering the expressed polypeptide from the transformant or the culture supernatant.

20 6. A method for screening a compound that inhibits the interaction between Calcineurin and NF-AT, the method comprising:

(a) contacting the polypeptide of claim 1 with Calcineurin in the presence or absence of a sample;

25 (b) detecting the binding activity of the polypeptide to Calcineurin; and

(c) selecting a compound that reduces the binding activity compared with the binding activity detected in the absence of the sample.

7. A compound isolable by the screening method of claim 6.

30 8. A pharmaceutical composition comprising the compound of claim 7 as an active ingredient.

9. A pharmaceutical composition comprising the polypeptide of claim 1 as an active ingredient.

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10.A method of suppressing immune, the method comprising administering the pharmaceutical composition of claim 8 to a patient in need of immunosuppression.

5 11.A method of suppressing immune, the method comprising administering the pharmaceutical composition of claim 9 to a patient in need of immunosuppression.

10 12.A method of preventing the hypertrophy of cardiac smooth muscle or vascular smooth muscle, the method comprising administering the pharmaceutical composition of claim 8 to a patient.

15 13.A method of preventing the hypertrophy of cardiac smooth muscle or vascular smooth muscle, the method comprising administering the pharmaceutical composition of claim 9 to a patient.

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**ABSTRACT**

Calcineurin (CN)-binding regions in the N-terminal domain derived from NF-ATx is disclosed. Also disclosed are CN-binding polypeptide compositions, DNA segments encoding these polypeptides, and methods of use. The CN-binding polypeptides bind to CN, suppressing the interaction between NF-AT and CN. The CN-binding polypeptide compositions can be used for treating pharmacological intervention with  $\text{Ca}^{2+}$ -dependent signaling events. The CN-binding polypeptide or DNA segments encoding them can be used to assay and screen candidates of pharmaceuticals, in particular, in the evaluation and characterization of immunosuppressants specifically interfering with the interaction between NF-AT and CN.

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Figure 1

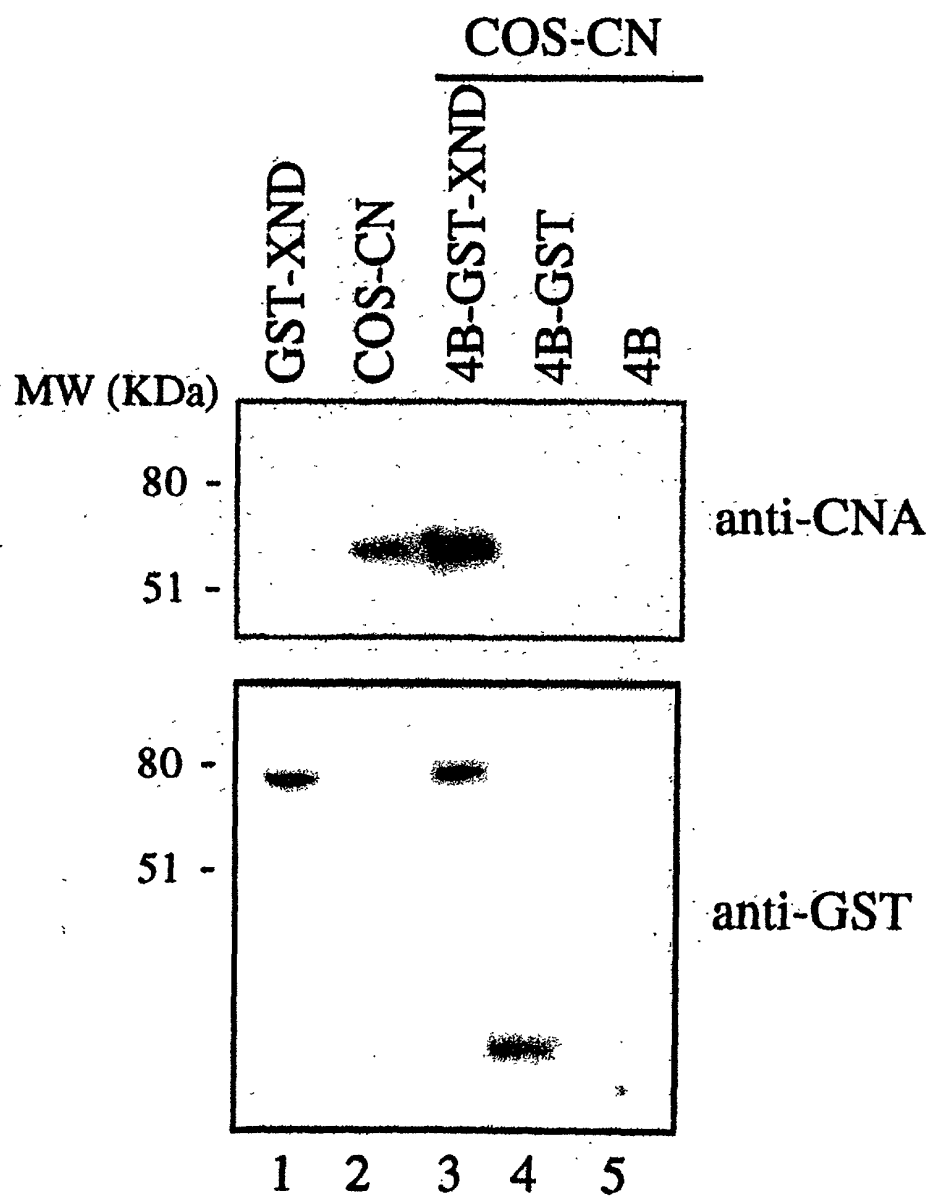


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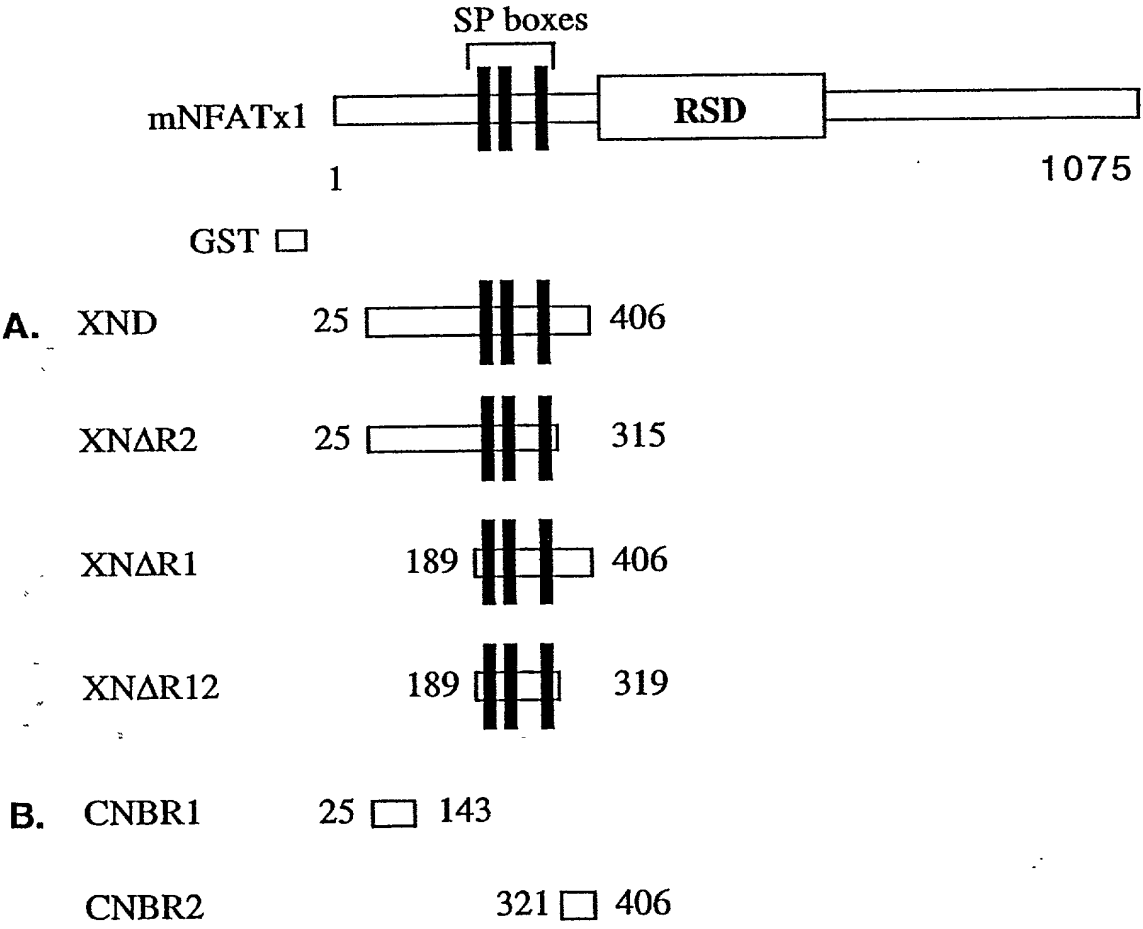


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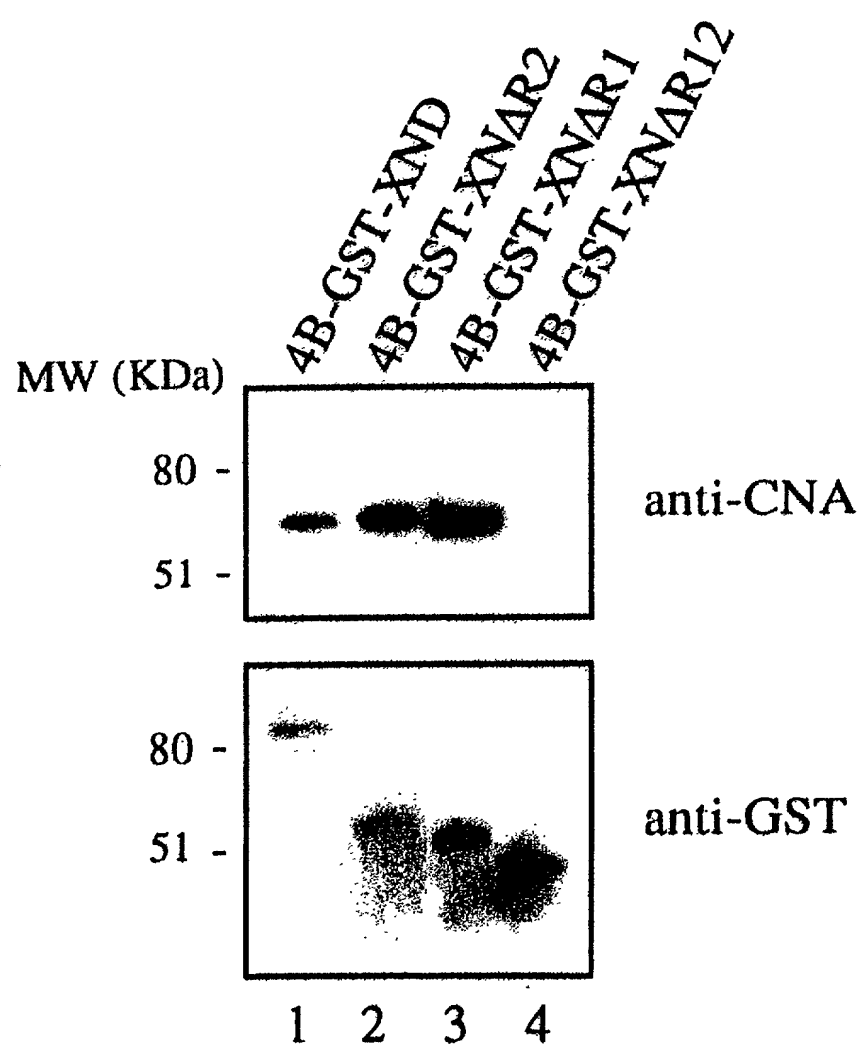


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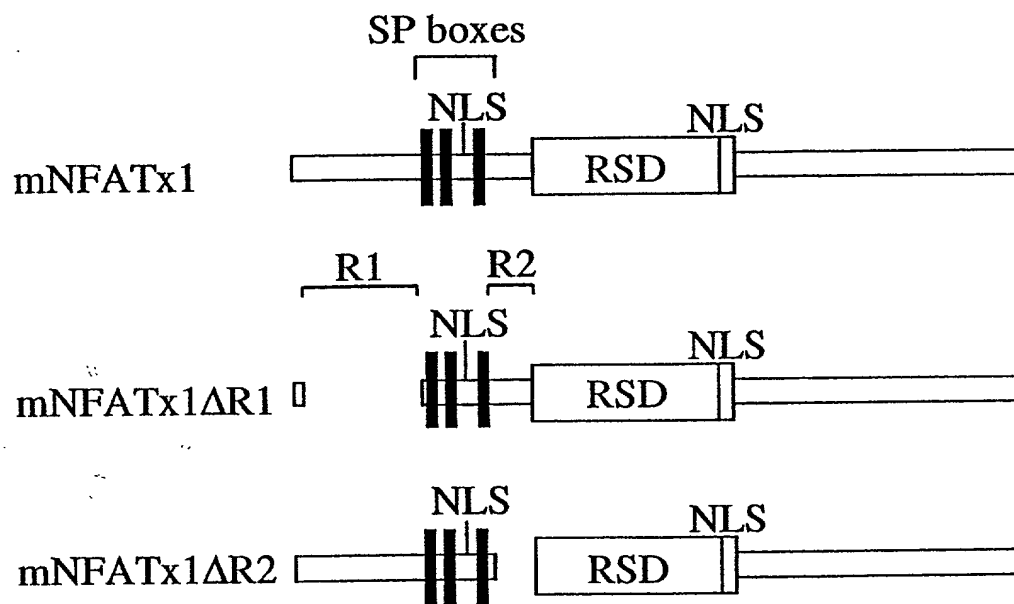


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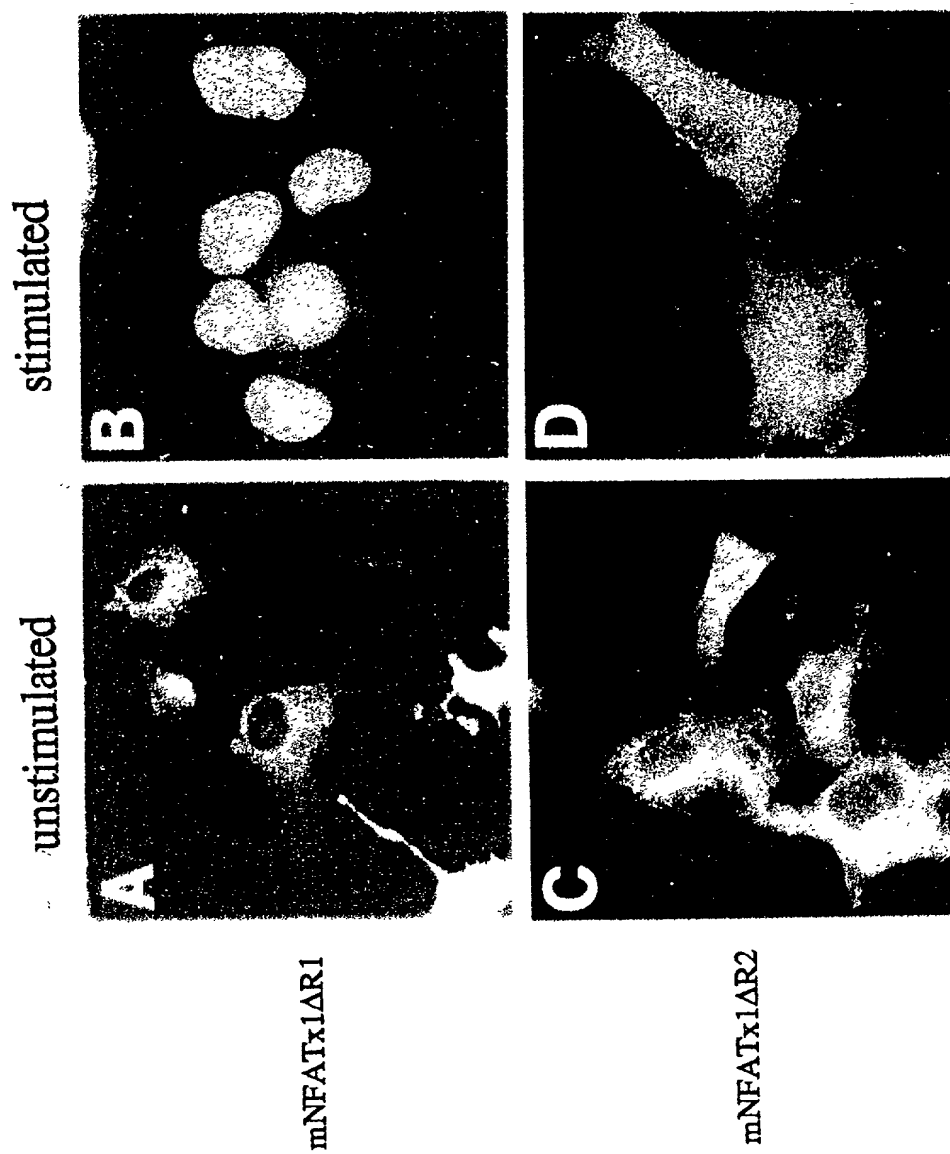




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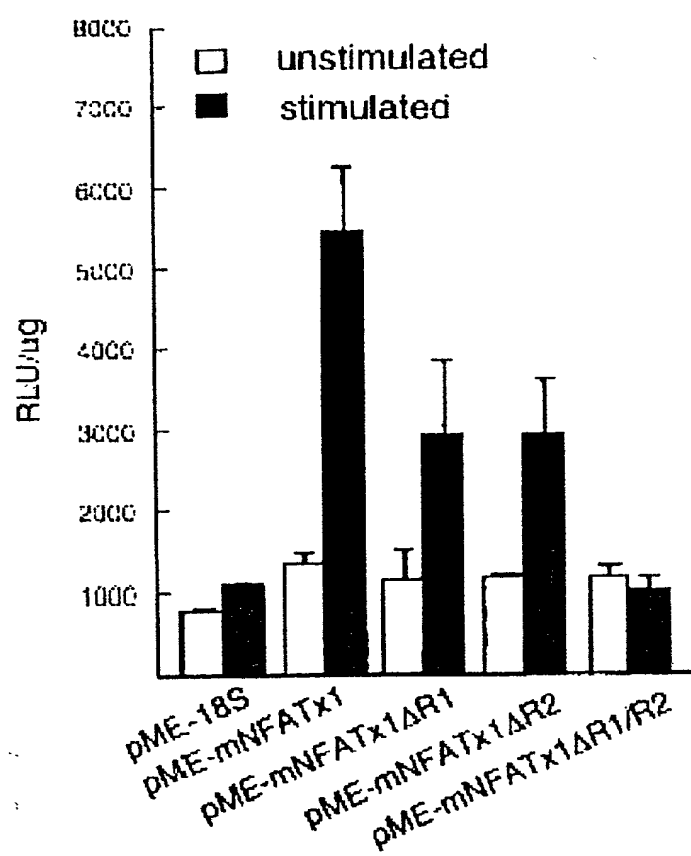
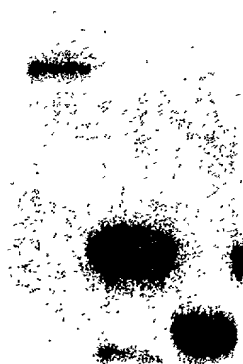


Figure 7

4B-GST-XND  
4B-GST-CNBR1  
4B-GST-CNBR2



anti-CNA



anti-GST

Figure 8

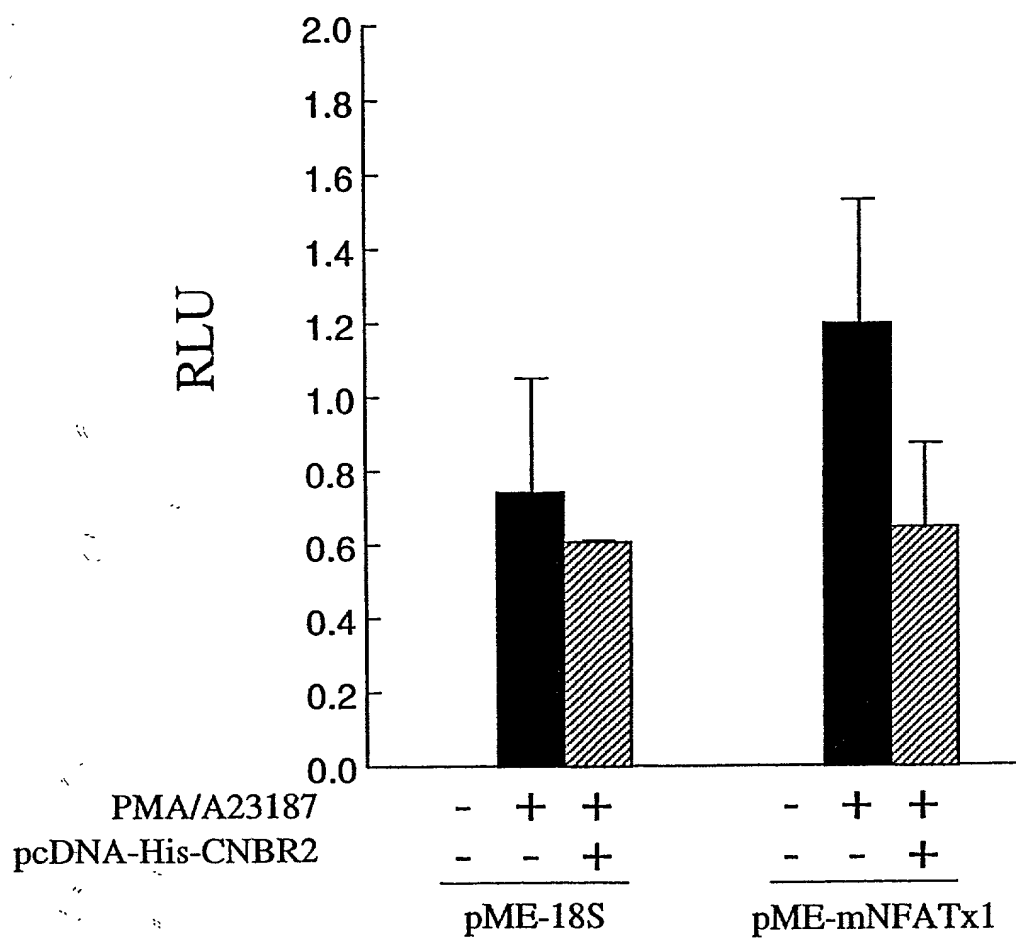


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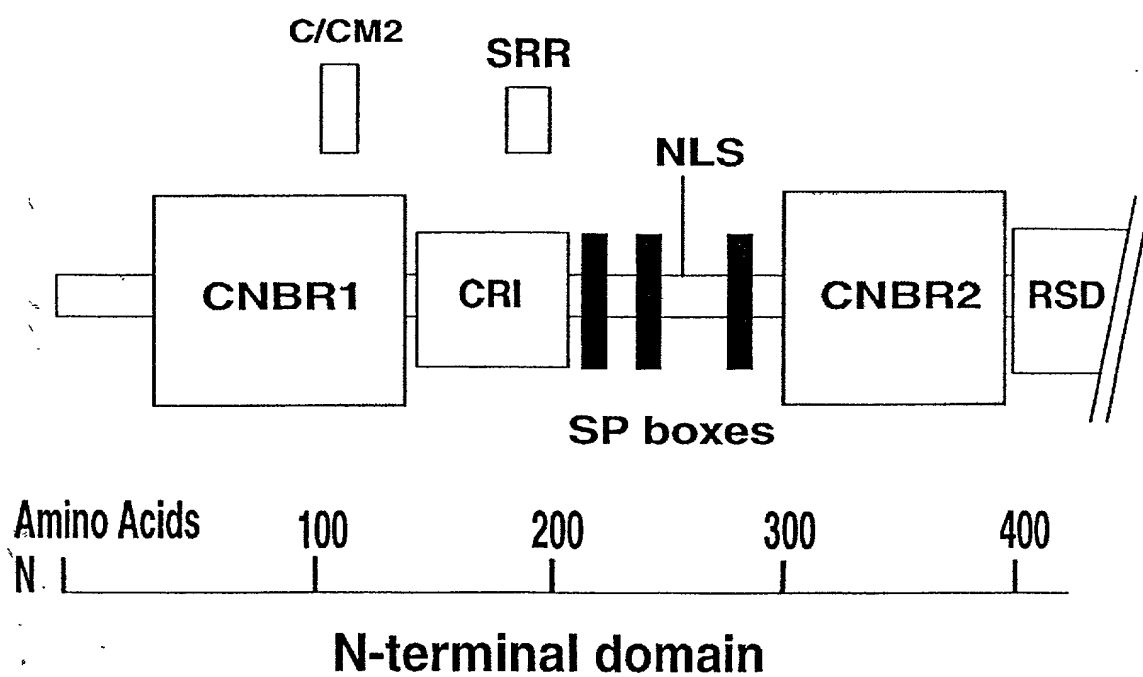


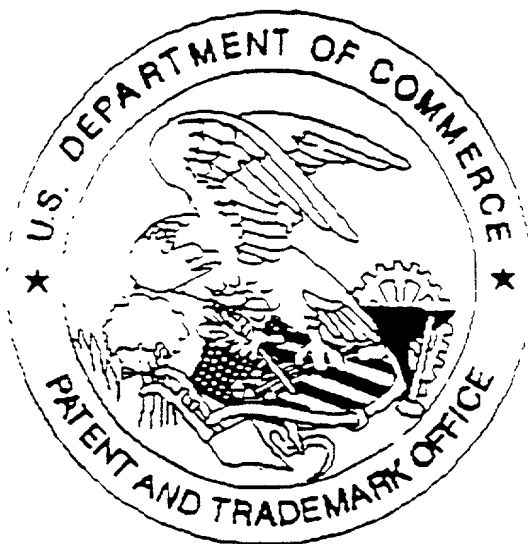
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hNFATx	1	.....	79
NFATc3	1	PRRVLFVSA QL.SRTR..PSDL.....NSS.G.....LQ.....	69
mNFATx	80	LQSHKNYEGTCEIPESKYSPLGGPKPFECPSIQFTSISPNCCQQLDAHEDDLQINDPEREFLEPSSRDHLYLPLEPSYRE	159
hNFATx	80	.....I.....H.....	159
NFATc3	70	..GY.....GD.S.....I.....H.GT.....H.....Y.....	149
mNFATx	160	SSLSPSPASSISSRSWFSASSCESLSHIYDDVDSELNEAAARFTLGSLTSPGGSPGGCPGEESWHQQYGGHSLSPRQ	239
hNFATx	160	.....	239
NFATc3	150	.....T.....L.....	229
mNFATx	240	SPCHSPRSSITDENWLSPPRASPSSRPTSPCGKRRHSSAEVCYAGSLSPHSPVSPGHSRPGSVTEDTWLTAPVHTGS	319
hNFATx	240	.....V.....	319
NFATc3	230	.....N.S..G..	309
mNFATx	320	GLSPAPFPFYQCVETDIPLKTRKTSQDAAILPGKLEICSDQDLSPSRETSDVDDGLGSQYPLKKDSSGDQFLSVPSPF	399
hNFATx	320	..G.V.....L.....S.A..I.....C.....	399
NFATc3	310	.....	389
mNFATx	400	TWSKPKRGHTPIFRTSSLPPLDWPLPTHFGQCELKIEVQPKTHRAHYETEGSRGAVKASTGGHPVVVKLLGYSEKPINLQ	479
hNFATx	400	.....A.....	479
NFATc3	390	.....N.....	469
mNFATx	480	MFIGTADDRYL RPHAFYQVHRITGKT VATASQEIIIASTKVLEIPLL PENNMSASIDCAGILKLRNSDIELRKGETDIGR	559
hNFATx	480	.....	479
NFATc3	470	.....	469

Figure 11

NPAT1	.....L.....G.....E.....P.....	.....P.....	65
NPAT2	.....L.....G.....E.....P.....	.....P.....	64
NPAT3	.....L.....G.....E.....P.....	.....P.....	60
NPAT1	.....L.....G.....E.....P.....	.....P.....	122
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NPAT1	.....L.....G.....E.....P.....	.....P.....	342
NPAT2	.....L.....G.....E.....P.....	.....P.....	355
NPAT3	.....L.....G.....E.....P.....	.....P.....	350
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NPAT2	.....L.....G.....E.....P.....	.....P.....	413
NPAT3	.....L.....G.....E.....P.....	.....P.....	404

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## SEQUENCE LISTING

<110> Arai, Ken-ichi  
Liu, Jie

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